LYOPHILISATION TECHNOLOGY FOR ISOLATION OF BLUEBERRY ANTHOCYANIN'S PATENT No. 288313/2015

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ABSTRACT. *Freeze-drying, also known as lyophilisation or cry desiccation, is a dehydration process typically used to preserve a perishable material or make the product - isolated natural substances minimize the effects of oxidation and other degradation processes and finally more convenient for transport. Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH medium. Generally they have the considerable preventive and therapeutic effects in relation to different diseases: anti-inflammation, antimicrobial, anti-tumor, anti-mutagenic and anti-oxidant pharmacological properties and a strong biological function. The aim of research and development is the optimization of lyophilisation process with acetone extracts of fruits of the northern high bush blueberry (Vaccinium corymbosum L.). Lyophilisation of anthocyanin extracts was carried out by the equipment GEA Lyophil SMART LYO SL2 at the Research and Development Department, Medicproduct, Co., Lipany, Slovakia. In regard to optimization of lyophilisation program - for each prepared extracts were tested and optimized various lyophilisating procedures with the most optimal process of lyophilisation in order to finally achieve a dry lyophilized powder. After lyophilization biological properties and the stability of anthocyanins were successfully tested.*

Key words: *Anthocyanins, Extract, High Bush Blueberry, Freeze-drying, Fruits.*

1. Introduction

Within the general separation of natural compounds (secondary metabolites of plants, animals and other organisms) distillation methods are used (hydro distillation or water vapour), which resulted in the extraction of volatile oils and extracts, where we get liquid and dry extracts. In both methods are used different types of solvents and higher temperature, which directly affects the stability and frequent breakdown of some sensitive natural components. In regard to this fact, lyophilisation is suitable to use for an isolation of special types of natural substances.

Freeze-drying, also known as lyophilisation or cry desiccation, is a dehydration process typically used to preserve a perishable material or make the product, natural substances, with a minimize of the oxidation effects and other degradation processes and finally more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase [1, 4].

The aim of research studies were to use and optimisation the lyophilisation technology, as a fundamental procedure, for processing acetone extracts of Northern high bush blueberry (*Vaccinium corymbosum* L.) fruits and isolation of pure anthocyanins.

Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH medium. These natural components occur in all tissues of higher plants, including leaves, stems, roots, flowers, and fruits. Generally they have the considerable preventive and therapeutic effects in relation to different diseases: anti-inflammation, antimicrobial, anti-tumor, anti-mutagenic and anti-oxidant pharmacological properties and a strong biological function.

In regard to the aim of our experimental work - the lyophilisation programs were tested for the anthocyanin extracts. These lyophilisation processes are gradually optimized so that the result of lyophilisation was a perfect dry lyophilized powder.

2. MATERIAL AND METHODS

The plant fruits of the bush blueberry (*Vaccinium corymbosum* L.) for the isolation of anthocyanins were collected from a large-scale cultivation of a special crop in the Kriva region. The locality is situated in North part of Slovakia, at altitude: 634 m above sea level, average temperature during the year is 6º C and precipitation is 895 mm. The soil is very acidic with pH up to 4.2.

1000 g of fresh plant material was macerated in the excess of acetone volume from 3 to 5 times . The filtrate was separated by vacuum aspirator and transferred to a separator funnel and mixed with a double volume of chloroform and shaken several times. The solution was stored in a refrigerator overnight at 4° C. The aqueous phase was separated in the boiling flask. Acetone and chloroform additives were evaporated in a vacuum evaporator at 38° C. Extracts of fruits were supplied frozen in a content of about 400 ml.

Lyophilisation of anthocyanin extracts was carried out by the equipment GEA Lyophil SMART LYO SL2. This new equipment is localised at the Research and Development Department, Medicproduct, Co., Lipany, Slovakia. The liquid material (4 ml) was filled on an automatic filling line (Flexicom Watson Marlow FPC 50W, Denmark) into vials (size 10R, diameter 24 mm, height 45 mm). Vials were closed after filling, with rubber stoppers (type no. V9355) to the position for lyophilisation and were loaded into the lyophilisator. After lyophilisation the stoppers were closed and capped on the line (edging aluminium stopper type no. 25345). The prepared product was suitable for further processing and distribution to other workplaces for further analysis and research.

In general, the lyophilisation has several steps:

Freezing

On a larger scale, freezing is usually done using a freeze-drying machine. In this step, it is important to cool the material below its triple point, the lowest temperature at which the solid and liquid phases of the material can coexist. This ensures that sublimation rather than melting will occur in the following steps. Larger crystals are easier to freeze-dry. To produce larger crystals, the product should be frozen slowly or can be cycled up and down in temperature. This cycling process is called annealing. However, in the case of food, or objects with formerly-living cells, large ice crystals will break the cell walls, resulting in the destruction of more cells, which can result in increasingly poor texture and nutritive content. In this case, the freezing is done rapidly, in order to lower the material to below its eutectic point quickly, thus avoiding the formation of ice crystals. Usually, the freezing temperatures are between −50 °C and −80 °C [2]. The freezing phase is the most critical in the whole freeze-drying process, because the product can be spoiled if badly done. Amorphous materials do not have a eutectic point, but they do have a critical point, below which the product must be maintained to prevent melt-back or collapse during primary and secondary drying [2].

Evacuation and primary drying

During the primary drying phase, the pressure is lowered (to the range of a few millibars), and enough heat is supplied to the material for the water to sublime. The amount of heat necessary can be calculated using the sublimating molecules' latent heat of sublimation. In this initial drying phase, about 95% of the water in the material is sublimated. This phase may be slow (can be several days in the industry), because, if too much heat is added, the material's structure could be altered.

In this phase, pressure is controlled through the application of partial vacuum. The vacuum speeds up the sublimation, making it useful as a deliberate drying process. Furthermore, a cold condenser chamber and/or condenser plates provide a surface(s) for the water vapor to re-solidify on. This condenser plays no role in keeping the material frozen; rather, it prevents water vapor from reaching the vacuum pump, which could degrade the pump's performance. Condenser temperatures are typically below −50 °C [1, 4]. It is important to note that, in this range of pressure, the heat is brought mainly by conduction or radiation; the convection effect is negligible, due to the low air density.

Secondary drying

The secondary drying phase aims to remove unfrozen water molecules, since the ice was removed in the primary drying phase. This part of the freeze-drying process is governed by the material's adsorption isotherms. In this phase, the temperature is raised higher than in the primary drying phase, and can even be above 0 °C, to break any physic-chemical interactions that have formed between the water molecules and the frozen material. Usually the pressure is also lowered in this stage to encourage desorption (typically in the range of microbars, or fractions of a Pascal). However, there are products that benefit from increased pressure as well. After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed. At the end of the operation, the final residual water content in the product is extremely low, around 1% to 4% [3].

3. RESULTS AND DISCUSSION

In regard to the organic solvent acetone, residues were determined after its evaporation from extracts by Hot space GC method (gas chromatography). The results of the determination of acetone residues are in interval from 0.06 to 0.13 % m/m. The extract does not contain any solvent residue.

The process of freeze-drying consists of the standard phases [1, 4]:

- Freezing - at atmospheric pressure,
- Evacuation - reducing pressure and primary drying - sublimation action - turning solid to gas and its dissipation from space of lyophilization,
- Secondary drying - removal of residual moisture at increased temperatures its dissipation from space of lyophilization.

The work on optimization of lyophilization consisted of two parts:

- Optimization of dilution of sample: because extracts from plant become after evaporation of solvents thick viscous liquids, for a successful lyophilization was necessary to dilute them with purified water in quality that declares the European Pharmacopoeia (*Aqua purificata* PhEur). The ration 1: 1 is optimal for an extract from northern high bush blueberry.
- Optimization of lyophilization program: for each prepared extracts were tested and optimized various lyophilisating procedures with the most optimal process of lyophilization (Table 1 and Fig. 1) in order to finally achieve a dry lyophilized powder.

Table 1. Optimize Lyophilisation Programme for Anthocyanin Extract of Bush blueberry

Section	Temperature (°C)	Vacuum (µBar)	Time (min.)	Step
1	5	0	1	Loading
2	-30	0	60	Freezing
3	-30	0	120	Freezing
4	-30	200	30	Evacuation
5	-5	200	420	Drying
6	-5	200	240	Drying
7	5	200	330	Drying
8	5	200	210	Drying
9	35	200	240	Drying
10	35	100	240	Drying
11	35	50	270	Drying

NOTE: The parameter of vacuum: 0 is shown in lyophilization. This value indicates that in a chamber was atmospheric pressure. Pressures shown in µbars means reduction of pressure in regard to atmospheric pressure.

In regards to our experiences, the optimal extraction method and the process of freeze-drying for obtaining pure anthocyans from fruits of the bush blueberry (*Vaccinium corymbosum* L.) were developed. After lyophilization biological properties (antimicrobial and antioxidant properties) and the stability of anthocyanins were successfully tested.

Freeze-drying is a relatively expensive process. The equipment is about three times as expensive as the equipment used for other separation processes, and the high energy demands lead to high energy costs. Furthermore, freeze-drying also has a long process time, because the addition of too much heat to the material can cause melting or structural deformations. Therefore, freeze-drying is often reserved for materials that are heat-sensitive, such as proteins, enzymes, microorganisms, and blood plasma [5, 7]. The low operating temperature of the process leads to minimal damage of these heat-sensitive products in our case the anthocyanins.

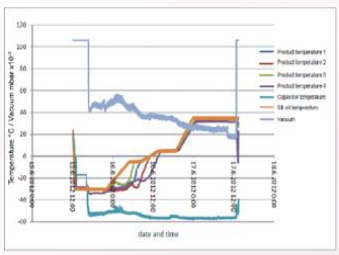

Fig. 1 The Courses of Temperature and Vacuum of Acetone Extract *Vaccinium corymbosum* L..

The Freeze-drying is used to preserve food, the resulting product being very lightweight [5]. Another example from the pharmaceutical industry is the use of freeze drying to produce tablets or wafers, the advantage of which is less excipient as well as a rapidly absorbed and easily administered dosage form. In chemical synthesis, products are often freeze-dried to make them more stable, or easier to dissolve in water for subsequent use. In bio separations, freeze-drying can be used also as a late-stage purification procedure, because it can effectively remove solvents. Furthermore, it is capable of concentrating substances with low molecular weights that are too small to be removed by a filtration membrane [7].

4. Conclusions

In regard to our experimental results, freeze-drying for obtaining pure anthocyans from fruits of the bush blueberry (*Vaccinium corymbosum* L.) had these characteristics the drying temperatures: from +5° C (a primary drying temperature) through -31° C (sublimation point) to +35° C (a secondary drying temperature), the maximum drying pressure: 200 µBar and total time of lyophilization was about 36 hours.

Our research work was done in cooperation with the pharmaceutical company Medicproduct, Co. in Lipany (Slovakia), which uses freeze-drying to increase the shelf life of products, such as vaccines and other injectable. By removing the water from the material and sealing the material in a vial, the material can be easily stored, shipped, and later reconstituted to its original form for injection. Our new original research deals with an optimize extraction and freeze-drying procedures in order to the natural components. The purpose is carrying out a dry, quality lyophilized product, which is then submitted to further analytical and biological testing.

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References

PATENTOVÁ LISTINA - PATENT číslo 288313/2015: Úrad priemyselného vlastníctva SR, Majiteľ: Medicproduct, a.s. Lipany, SK, Pôvodcovia: TOMASH, J. – FEJÉR, J. – MARiYCHUK, R. – ŠALAMON, I. – ŠIMKO, V., Názov: Spôsob lyofilizácie antokyánov z extraktov drobných plodov liečivých rastlín,

Opis: Vynález sa týka spôsobu lyofilizácie antokyánov z extraktov drobných plodov liečivých rastlín, pričom týmto postupom získané antokyány z plodov sa dajú priemyselne využiť v medicíne a farmácii (terapeutický), ďalej vo výžive (výživové doplnky) a ďalších oblastiach potravinárskeho priemyslu (aditíva).